Genomics 55, 135–146 (1999)
Article ID geno 1998 5513, available online at http://www.idealibrary com on IDEAL®

A Human Mitochondrial DNA Standard Reference Material for Quality Control in Forensic Identification, Medical Diagnosis, and Mutation Detection

Barbara C. Levin,*,[1] Haiyan Cheng,† and Dennis J. Reeder*

*Biotechnology Division, Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899; and †GEO-CENTERS, Inc., Newton Centre, Massachusetts 02159

Received May 8, 1998, accepted August 4, 1998

A human mitochondrial DNA (mtDNA) standard reference material (SRM 2392) will provide quality control when mtDNA is sequenced for forensic identifications, medical diagnosis, or mutation detection. SRM 2392 includes DNA from two lymphoblast cell cultures (CHR and 9947A) and cloned DNA from the CHR HV1 region, which contains a C stretch and is difficult to sequence. The mtDNA sequence (but not the DNA) of a third human template GM03798 is provided for comparison. Fifty-eight unique primer sets allow any area or the entire mtDNA (16,569 bp) to be amplified and sequenced. While none of the differences in these three templates correspond to published mutations associated with specific diseases, some of these differences did result in animo acid changes compared with that published by S. Anderson *et al.* (1981, *Nature* 290: 457–465). An interlaboratory evaluation of the amplification, sequencing, and data analysis of the CHR template was conducted by four laboratories. Corroboration of the SRM results will provide quality assurance that any unknown mtDNA is also being amplified and sequenced correctly. © 1999 Academic Press

[1] To whom correspondence should be addressed at 100 Bureau Drive, Stop 8311, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8311 Telephone (301) 975-6682. Fax (301) 330-3447. E-mail barbara levin@nist.gov.

INTRODUCTION

Human mitochondrial DNA (mtDNA) has been completely sequenced and found to be circular double-stranded molecules containing 16,569 bp (Anderson *et al.*, 1981). Each human cell can have a few dozen to several thousand molecules of mtDNA (Bogenhagen and Clayton, 1974; King and Attardi, 1989). Sequence analysis of mtDNA is being used by the forensic community for human identification, especially in those cases in which genomic DNA is highly degraded or nonexistent (Holland *et al.*, 1993, 1995). Forensic analysis to determine the distinction between individuals is primarily based on the considerable sequence variation found in the two hypervariable regions (HV1, HV2) located in the noncoding displacement loop (D loop). The medical community is also using sequence analysis of mtDNA for diagnoses of diseases associated with specific mutations and deletions (Wallace *et al.*, 1997). A third area of research, which is largely unexplored and which needs sequence analysis, is the examination of the mutagenic effects of chemical and physical agents on mtDNA (Grossman, 1995; Ballinger *et al.*, 1996). The objective of this research was to develop a human mtDNA standard reference material (SRM) for quality control in sequencing, forensic identifications, medical diagnostics, and mutation detection.

MATERIALS AND METHODS

Origin of extracted DNA. The DNA template designated CHR came from human white blood cells that were transformed with the Epstein–Barr virus and immortalized as a cell culture line (CHR cells) by the American Type Culture Collection (ATCC, Rockville, MD) After transformation. the cells were grown in Iscove's modified Dulbecco's media or RPMI 1640 media with L-glutamine, sodium bicarbonate, penicillin, streptomycin, and 20% fetal calf serum (Life Technologies, Inc., Grand Island, NY). The cell cultures were grown at 37°C in humidified atmosphere containing 5% CO_2 and 95% air The DNA was extracted from 2×10^8 CHR cells by the Qiagen Plasmid/Cosmid Purification Protocol (Qiagen, Inc., Chatsworth, CA). This procedure enhanced the concentration of mtDNA and reduced, but did not eliminate, nuclear DNA

The CHR data presented in this paper were obtained primarily with the above-mentioned immortalized CHR cell culture line. How-

0888-7543/99 $30 00

ever, before production of the final SRM began, it was necessary to obtain fresh blood from CHR and to reestablish the cell line. This second CHR cell line was established by the ATCC as above. The sequence of this second CHR cell line was examined and found to be identical to that of the first CHR cell line, with the single exception that no heteroplasmy was noted at bp 6849, the second CHR cell line agreed with Anderson at bp 6849. It is the second CHR cell line that is included in SRM 2392.

The DNA template 9947A was obtained from Life Technologies, Inc., who prepared it from a Epstein–Barr virus-immortalized human lymphoid cell line. DNA from 9947A is also used in the PCR-based DNA profiling standard (SRM 2391)[2] designed for forensic and paternity testing, law enforcement training, and research.

A third DNA template was extracted from an apparently normal human lymphoblastoid cell culture (GM03798) obtained from NIGMS[3] and grown in the same manner as the CHR cells. The DNA was extracted using DNA NOW, a phenol-free DNA isolation reagent (BIOGENTEX, Seabrook, TX). The information on this template is included for informational purposes only; the DNA is not included as part of this SRM.

Isolation and cloning of mtDNA containing the C stretch. Confluent CHR cells were harvested by centrifugation at 1500 rpm for 5 min. The mtDNA was isolated using the Qiagen Plasmid/Cosmid Purification Protocol (Qiagen, Inc.). Following isolation, the mtDNA was digested with restriction enzymes *Sac*I and *Kpn*I (New England Biolabs, Inc., Beverly, MA) into five fragments which were separated on a 0.7% low-melting-agarose gel. Bands of the size of the fragment containing the HV1 region were cut from the gel and melted at 65°C. DNA was extracted with phenol twice and precipitated by adding sodium chloride (150 mM) and 2 vol of 100% ethanol. The final product was resuspended in Tris–EDTA (TE) buffer.

The cloning vector, M13mp18, was also digested with *Sac*I and *Kpn*I, treated with calf intestinal alkaline phosphatase (New England Biolabs, Inc.), extracted with phenol, and precipitated with NaCl and ethanol as described above. The vector was incubated with the mtDNA product and T_4 DNA ligase (Life Technologies, Inc.) at 4°C overnight. An overnight culture of *Escherichia coli* host TG-1 cells was diluted and grown at 37°C in LB media (Sambrook *et al.*, 1989) until the OD_{650} reached 0.4–0.5. The cells were harvested by centrifugation at 1500 rpm for 5 min. The cell pellet was resuspended in 10 ml calcium chloride (50 mM) and incubated for 1 h on ice, centrifuged, and resuspended in 1 ml calcium chloride (50 mM) and incubated for 30 min on ice. The treated TG-1 cell suspension (0.3 ml) was incubated for 30 min on ice with 20 μl of a ligation mixture containing the isolated mtDNA fragment, the cloning vector that had been treated overnight, T_4 ligase, and ligation buffer (Life Technologies, Inc.). This mixture was then exposed to a heat shock of 42°C for 2 min; mixed with 0.2 ml of untreated TG-1 cells (from the overnight culture), 4 μl of 1 M isopropylthio-β-D-galactoside, 40 μl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 3 ml of melted (55°C) top agar, and spread on the surface of freshly prepared LB agarose plates (Sambrook *et al.*, 1989). The plates were incubated at 37°C overnight. Both colorless and blue plaques were visible in the morning. The colorless plaques indicate that insertion of the vector has occurred, whereas the blue plaques have no insertion of the vector.

Bacteriophage DNA isolation and sequencing. Single, well-isolated colorless plaques from the above LB plates were each placed in a sterile tube with 1.5 ml of a TG-1/LB cell suspension that contained TG-1 cells that were grown overnight and diluted 1/100 in LB media and grown for 1 h at 37°C. The plaques and the TG-1/LB cell suspension were grown at 37°C for 5 h. Cell debris was removed by centrifugation at 15,000 rpm for 5 min. The supernatant containing the bacteriophage was incubated with 0.2 ml polyethylene glycol (20% PEG in 2.5 M NaCl) overnight at 4°C, and the resultant precipitate containing the DNA was pelleted by centrifugation at 15,000 rpm for 15 min. The bacteriophage DNA was isolated by phenol extraction and NaCl/ethanol precipitation as described above and then dissolved in 25 μl of TE buffer. The bacteriophage DNA was cycle sequenced with AmpliTaq DNA polymerase, FS, and the −21 M13 primer: 5′-TGTAAAACGACGGCCAGT-3′ according to the protocol in the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Foster City, CA). The cycle sequencing was conducted in a Perkin–Elmer Model 9600 thermocycler by first heating the DNA reaction mixture at 96°C for 1 min and then subjecting the mixture to 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min. The cycle sequencing product was purified using a Centri-Sep spin column (Princeton Separations, Inc., Adelphia, NJ). The DNA pellet was rinsed with 70% ethanol, vacuum dried, resuspended in loading buffer prepared by combining deionized formamide and 25 mM EDTA (pH 8.0) in a ratio of 5:1, loaded onto a 4.75% acrylamide gel, and electrophoresed on an ABI 373 DNA sequencer. One of the clones containing the C stretch sequence was used as the source of the cloned DNA for the SRM.

mtDNA primers. Fifty-eight sets of unique primers (19–28 bp) for sequencing the entire mtDNA (16,569 bp) were computer-designed using Gene Runner for Windows (Hastings Software, Inc., Hastings, NY) and custom made by Bio-Synthesis, Inc. (Lewisville, TX). The −21M13 primer was used to sequence the cloned HV1 region of the DNA from the CHR template. The sequences of all the primers are shown in Table 1.

Polymerase chain reaction (PCR). Extracted DNA was resuspended in TE buffer (pH 7.5) containing 10 mM Tris and 1 mM EDTA. The PCR mixture contained DNA (1 μl), *Taq* DNA polymerase (0.5 μl or 2.5 units) (Boehringer Mannheim), and 10× buffer (5 μl) (Boehringer Mannheim), dNTP's (0.2 mM each) (Life Technologies, Inc.), forward and reverse primers (0.4 μM each), plus H_2O to a final volume of 50 μl. The 10× buffer (pH 8.3) contained Tris–HCl (100 mM), $MgCl_2$ (15 mM), and KCl (500 mM).

Thermal cycling was conducted in a Perkin–Elmer Model 9600 thermocycler and consisted of 1 min at 96°C; followed by 32 cycles of 15 s at 94°C (denaturation), 30 s at 56°C (annealing), and 15 s at 72°C (extension); and ending with a final extension of 7 min at 72°C.

A sample of the amplified DNA was electrophoresed in 0.7% agarose and stained with ethidium bromide to assess the purity and size of the PCR product. Before sequencing, extraneous materials were removed from the PCR product with a QIAquick PCR Purification Kit (QIAGEN, Inc.).

Sequencing. Cycle sequencing using fluorescent dye-labeled terminators was performed with an ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (Perkin–Elmer, Foster City, CA). Thermal cycling was conducted in a Perkin–Elmer Model 9600 thermocycler and started with 1 min at 96°C. The reaction then underwent 25 cycles of 96°C for 15 s (denaturation), 50°C for 5 s (annealing), and 60°C for 2 min (extension). The DNA product was purified by passage through a Centri-Sep spin column (Princeton Separations, Inc.).

Electrophoresis and sequencing of the fluorescently labeled purified DNA were performed with a 373 ABI Sequencer (Perkin–Elmer) using a 4.75% acrylamide gel. Data analysis was executed with the Sequence Navigator software package (Perkin–Elmer).

Interlaboratory evaluation. Three laboratories in addition to NIST participated in an interlaboratory evaluation of the CHR template. These laboratories were The Bode Technology Group, Inc. (21515 Ridgetop Circle, Suite 140, Sterling, VA 20166), IIT Research Institute (Virginia Technology Center, 8510 Cinderbed Road, Suite 300, P.O. Box 899, Newington, VA 22122), and Lark Technologies, Inc. (9545 Katy Fwy, Suite 465, Houston, TX 77024).

Each laboratory was sent:

1. Two tubes of DNA from the first CHR cell culture line. One tube contained extracted DNA ready for PCR amplification of the entire mtDNA. The other contained the cloned DNA ready for cycle sequencing of the HV1 region (this DNA did *not* need to be PCR amplified).

[2] SRM 2391 may be obtained from the Standard Reference Material Program, NIST, Gaithersburg, Maryland 20899.

[3] NIGMS Human Genetic Mutant Cell Repository, Cornell Institute for Medical Research, 401 Haddon Avenue, Camden, New Jersey 08103.

2. Fifty-eight sets of primers labeled with either F# (forward primer) or R# (reverse primer) Forward and reverse primers with the same number were paired and numbered from the 5′ end. Primers were diluted to 10 μl and ready for use. Also enclosed was the −21M13 primer for the sequencing of the cloned HV1 region of the CHR DNA, which covered basepairs 16133 to 40.

3. The protocol used at NIST to amplify and sequence the DNA. The laboratories, however, were free to use any protocol with which they were familiar and felt comfortable

4. A form table to record the results This table provided the number of the primer set, the region that each primer set amplified, and the length of the amplified region. We requested that the laboratory fill in the differences found when they compared the sequence that they determined for the SRM with that of Anderson *et al.* (1981).

5. The following cautionary note

> WARNING. The DNA and cells were derived from a cell culture line from an apparently healthy human subject. The cell culture line has been tested and found to be nonreactive for hepatitis B surface antigen and HIV However, no test method can ensure that a product derived from human blood does not contain HIV, hepatitis or other infectious agents. HANDLE AS IF CAPABLE OF TRANSMITTING DISEASE. (The second CHR cell culture line generated from the same individual was not tested again for hepatitis or HIV. Normal precautions should be used)

Differences in methodology used by laboratories in interlaboratory evaluation. The Bode Technology Group, Inc., essentially followed the NIST protocol except that they used a 6% acrylamide/8.3 M urea gel for the sequencing electrophoresis instead of a 4.75% acrylamide.

IIT Research Institute also followed the NIST protocol except that they used *Taq* Gold (Perkin–Elmer) for the amplification reaction, which was modified to include a hot start of 95°C for 11 min. Microcon 100 microconcentrators (Amicon, Inc., Beverly, MA) were used to purify the PCR products. The quantities of DNA were determined by capillary electrophoresis (CE) with a Beckman P/ACE 5010 System (Beckman Instruments, Inc., Fullerton, CA) as follows 1 μl of the amplified product was mixed with 25 μl of sterile deionized H_2O containing 0 52 ng/μl of a 200-bp internal standard (GenSura Laboratories, Inc , Del Mar, CA) and run on the CE. One determines the quantity of the amplified product from the ratio of the PCR product peak area to the internal standard peak area multiplied by a migration standard. A 6% acrylamide gel was used for the sequencing electrophoresis instead of a 4.75% acrylamide

Lark Technologies, Inc., followed the NIST protocol with the following differences: AmpliTaq DNA polymerase (Perkin–Elmer) was used to amplify the DNA; the dNTP's were purchased from Pharmacia Biotech, Inc (Piscataway, NJ); the products were purified with Qiaquick PCR purification kit (Qiagen); in the sequencing reactions, the amount of PCR product used varied from 1 to 3 μl based on the concentration estimated from agarose gels; cycling conditions were 95°C for 1 min followed by 25 cycles of 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min, the sequence reactions were cleaned up by ethanol precipitation, a 4 25% polyacrylamide gel was used for the sequencing electrophoresis instead of 4.75% acrylamide; and an ABI 377 was used instead of the ABI 373 Electropherograms were printed for each reaction, and the sequences were manually edited based on the electropherogram patterns. Printed electropherograms and a floppy disk with the sequence data were sent to NIST where the data were compared to the Anderson sequence.

RESULTS AND DISCUSSION

SRM templates. Two DNA templates, CHR and 9947A, are included in the NIST human mtDNA sequencing SRM 2392. Both of these DNA samples come from human cell culture lines that were developed from apparently normal individuals. The DNA from 9947A is the total extracted DNA, which also includes nuclear DNA. The DNA from CHR was isolated in a manner that enhanced the concentration of the mtDNA, but did not totally eliminate the nuclear DNA. The SRM also provides cloned DNA from the HV1 region of the CHR template, which contains a C stretch. In most people, the HV1 region has a string of cytosine (C) residues interrupted by a thymine (T) at nucleotide position 16189.[4] In some individuals, however, a transition that changes the T to a C occurs, producing a long string of Cs called the C stretch. When this happens, sequencing beyond the C stretch becomes very difficult, if not impossible. Clones of the HV1 region containing the C stretch indicated that the number of Cs differed among the different clones and the difficulty in sequencing was due to frameshifts that resulted from the simultaneous sequencing of templates with differing numbers of Cs (Bendall and Sykes, 1995; Levin *et al.*, 1995, 1997). We found, however, that one could sequence through the entire HV1 region including the C stretch without problems if one used the clone of the area. Therefore, we have included the cloned HV1 region of the CHR DNA template in the SRM.

In addition to templates CHR and 9947A, we have included all the information regarding a third template from a lymphoblastoid cell line (GM03798) that was obtained from the NIGMS Human Genetic Mutant Cell Repository and that was completely sequenced at NIST three times. The DNA from this cell line is not part of the SRM, and the data are included for information only.

Primers. The 58 sets of unique primers were designed to allow the amplification and sequencing of any region or the entire 16,569 bp that comprise human mitochondrial DNA. The sequences of both the forward and the reverse primers that are in each set are shown in Table 1. The numbers indicate the 5′ end of the primer. They are all between 19 and 28 bp long, and the criteria that were used to choose these primers were primer T_m = 50–65°C, primer length = 15–30 bp, and PCR product length = 400–850 bp. The primers were designed to produce sequences that overlapped with both the previous and the following regions to allow those areas in the beginning and end of electropherograms, which are difficult to sequence, to become readable. Table 2 shows the number of the base where the readable sequence begins and ends (indicated in the table as "start" and "end"). The readable region is always smaller than the amplified region.

In addition to the designed primers, we also used the −21M13 primer (Table 1) to sequence the cloned DNA from the HV1 region of the CHR template that contained the C stretch. The PCR products produced single, distinct bands for all 58 primer sets (Figs. 1A and 1B).

Differences between the SRM templates and the Anderson sequence. Anderson and his co-workers completely sequenced human mtDNA in 1981 (Anderson *et al.*, 1981). All investigators who subsequently examined human mtDNA have used the numbering system of Ander-

[4] All nucleotide numbers referred to in this paper are based on the numbering system of Anderson *et al.* (1981)

TABLE 1

Primer Sets Used for PCR Amplification of Human mtDNA

Primer set		Primer sequence	Primer set		Primer sequence
1 (HV2)	F15	CACCCTATTAACCACTCACG		R8215	GACGATGGGCATGAAACTG
	R484	TGAGATTAGTAGTATGGGAG	31	F7901	TGAACCTACGAGTACACCGACTAC
2	F361	ACAAAGAACCCTAACACCAGC		R8311	AAGTTAGCTTTACAGTGGGCTCTAG
	R921	ACTTGGGTTAATCGTGTGACC	32	F8164	CGGTCAATGCTCTGAAATCTGTG
3	F756	CATCAAGCACGCAGCAATG		R8669	CATTGTTGGGTGGTGATTAGTCG
	R1425	AATCCACCTTCGACCCTTAAG	33	F8539	CTGTTCGCTTCATTCATTGCC
4	F873	GGTTGGTCAATTTCGTGCCAG		R9059	GTGGCGCTTCCAATTAGGTG
	R1425	AATCCACCTTCGACCCTTAAG	34	F8903	CCCACTTCTTACCACAAGGC
5	F1234	CTCACCACCTCTTGCTCAGC		R9403	GTGCTTTCTCGTGTTACATCG
	R1769	GCCAGGTTTCAATTTCTATCG	35	F9309	TTTCACTTCCACTCCATAACGC
6	F1587	TGCACTTGGACGAACCAGAG		R9848	GAAAGTTGAGCCAATAATGACG
	R2216	TGTTGAGCTTGAACGCTTTC	36	F9449	CGCGATAATCCTATTTATTACCTCAG
7	F1657	CTTGACCGCTCTGAGCTAAAC		R9995	AGAGTAAGACCCTCATCAATAGATGG
	R2216	TGTTGAGCTTGAACGCTTTC	37	F9754	AGTCTCCCTTCACCATTTCCG
8	F1993	AAACCTACCGAGCCTGGTG		R10275	AAAGGAGGGCAATTTCTAGATC
	R2216	TGTTGAGCTTGAACGCTTTC	38	F10127	ACTACCACAACTCAACGGCTAC
9	F2105	GAGGAACAGCTCTTTGGACAC		R10556	GGAGGATATGAGGTGTGAGCG
	R2660	AGAGACAGCTGAACCCTCGTG	39	F10386	GGATTAGACTGAACCGAATTGG
10	F2417	CACTGTCAACCCAACACAGG		R11166	CATCGGGTGATGATAGCCAAG
	R3006	ATGTCCTGATCCAACATCGAG	40	F10704	GTCTCAATCTCCAACACATATGG
11	F2834	CCCAACCTCCGAGCAGTACATG		R11267	TGTTGTGAGTGTAAATTAGTGCG
	R3557	AGAAGAGCGATGGTGAGAGC	41	F11001	AACGCCACTTATCCAGTGAACC
12	F2972	ATAGGGTTTACGACCTCGATG		R11600	CTGTTTGTCGTAGGCAGATGG
	R3557	AGAAGAGCGATGGTGAGAGC	42	F11403	GACTCCCTAAAGCCCATGTCG
13	F3234	AGATGGCAGAGCCCGGTAATC		R11927	TTGATCAGGAGAACGTGGTTAC
	R3557	AGAAGAGCGATGGTGAGAGC	43	F11760	ACGAACGCACTCACAGTCG
14	F3441	ACTACAACCCTTCGCTGACG		R12189	AAGCCTCTGTTGTCAGATTCAC
	R3940	TGAAGCCTGAGACTAGTTCGG	44	F11901	TGCTAGTAACCACGTTCTGGTG
15	F3635	GCCTAGCCGTTTACTCAATCC		R12876	GATATCGCCGATACGGTTG
	R4162	TGAGTTGGTCGTAGCGGAATC	45	F12357	AACCACCCTAACCCTGACTTCC
16	F3931	TCAGGCTTCAACATCGAATACG		R12876	GATATCGCCGATACGGTTG
	R4728	TTATGGTTCATTGTCCGGAGAG	46	F12601	TTCATCCCTGTAGCATTGTTCG
17	F4183	TTTCTACCACTCACCCTAGCATTAC		R13123	AGCGGATGAGTAAGAAGATTCC
	R4728	TTATGGTTCATTGTCCGGAGAG	47	F12793	TTGCTCATCAGTTGATGATACG
18	F4392	CCCATCCTAAAGTAAGGTCAGC		R13343	TTGAAGAAGGCGTGGGTACAG
	R4983	GGTTTAATCCACCTCAACTGCC	48	F13188	CACTCTGTTCGCAGCAGTATG
19	F4447	TTGGTTATACCCTTCCCGTAC		R13611	TCGAGTGCTATAGGCGCTTGTC
	R4982	GTTTAATCCACCTCAACTGCC	49	F13518	CATCATCGAAACCGCAAAC
20	F4797	CCCTTTCACTTCTGAGTCCCAG		R13935	TGTGATGCTAGGGTAGAATCCG
	R5553	AGGGCTTTGAAGGCTCTTG	50	F13715	GAAGCCTATTCGCAGGATTTC
21	F4976	ATTAAACCAGACCCAGCTACG		R14118	TGGGAAGAAGAAAGAGAGGAAG
	R5553	AGGGCTTTGAAGGCTCTTG	51	F13899	TTTCTCCAACATACTCGGATTC
22	F5318	CACCATCACCCTCCTTAACC		R14388	TTAGCGATGGAGGTAGGATTCG
	R5882	GCTGAGTGAAGCATTGGACTG	52	F14189	ACAAACAATGGTCAACCAGTAAC
23	F5700	TAAGCACCCTAATCAACTGGC		R14926	TGAGGCGTCTGGTGAGTAGTGC
	R6262	GCCTCCACTATAGCAGATGCG	53	F14470	TCCAAAGACAACCATCATTCC
24	F5999	TCTAAGCCTCCTTATTCGAGC		R14996	CGTGAAGGTAGCGGATGATTC
	R6526	ATAGTGATGCCAGCAGCTAGG	54	F14909	TACTCACCAGACGCCTCAACCG
25	F6242	CGCATCTGCTATAGTGGAGG		R15396	TTATCGGAATGGGAGGTGATTC
	R6526	ATAGTGATGCCAGCAGCTAGG	55	F15260	AGTCCCACCCTCACACGATTC
26	F6426	GCCATAACCCAATACCAAACG		R15774	ACTGGTTGTCCTCCGATTCAGG
	R7030	TGGGCTACAACGTAGTACGTG	56	F15574	CGCCTACACAATTCTCCGATC
27	F6744	GGCTTCCTAGGGTTTATCGTG		R16084	CCGTTGTTGATGGGTGAGTC
	R7255	TTTCATGTGGTGTATGCATCG	57 (HV1)	F15971	TTAACTCCACCATTAGCACC
28	F7075	GAGGCTTCATTCACTGATTTCC		R16451	GCGAGGAGAGTAGCACTCTTC
	R7792	GGGCAGGATAGTTCAGACGG	58	F16097	TACATTACTGCCAGCCACCATG
29	F7215	CGACGTTACTCGGACTACCC		R336	TTAAGTGCTGTGGCCAGAAG
	R7792	GGGCAGGATAGTTCAGACGG	−21M13	F	TGTAAAACGACGGCCAGT
30	F7645	TATCACCTTTCATGATCACGC			

son and have compared their sequence findings to those described by Anderson. However, the DNA sequenced by Anderson is not available for use as a positive control during actual experiments, whereas NIST SRM 2392 would be available. Table 2 shows the mtDNA differences compared to the Anderson sequence that were found at NIST with all three templates—CHR, 9947A, and GM03798. In all three templates, all 58 areas comprising

TABLE 2

Primer Sets Used for PCR Amplification of Human mtDNA and Differences with the Anderson Sequence Found in Three Templates at NIST

Primer set	Amplified region[a]	Length of amplified region	Comparison with Anderson: Anderson No.	Anderson bp	Template CHR	Template 9947A	Template GM03798	Amino acid change
1 (HV2)	15–484	470			Start 39	Start 39	Start 55	
			73	A	G	—	—	
			93	A	—	G	—	
			195	T	C	C	—	
			204	T	C	—	—	
			207	G	A	—	—	
			214	A	—	G	—	
			263	A	G	G	G	
			309 1		C(ins)	C(ins)	—	
			309 2		—	C(ins)	—	
			315 1		C(ins)	C(ins)	C(ins)	
					End 436	End 473	End 454	
2	361–921	561			Start 429	Start 421	Start 415	
			709	G	A	—	A	
			750	A	G	G	G	
					End 891	End 846	End 834	
3	756–1425	670	None		Start 778	Start 778	Start 818	
					End 1197	End 1278	End 1146	
4	873–1425	553	None		Start 931	Start 928	Start 938	
					End 1335	End 1377	End 1323	
5	1234–1769	536			Start 1279	Start 1275	Start 1295	
			1438	A	G	G	G	
			1719	G	A	—	E	
					End 1738	End 1741	End 1654	
6	1587–2216	630			Start 1632	Start 1632	Start 1649	
			1719[b]	G	A	—	—	
					End 2106	End 2106	End 2031	
7	1657–2216	560			Start 1691	Start 1686	Start 1715	
			1719[b]	G	A	—	—	
					End 2170	End 2173	End 2097	
8	1993–2216	224	None		Start 2036	Start 2018	Start 2069	
					End 2213	End 2217	End 2212	
9	2105–2660	556	None		Start 2157	Start 2150	Start 2161	
					End 2636	End 2586	End 2560	
10	2417–3006	590			Start 2465	Start 2458	Start 2483	
			2706	A	G	—	—	
					End 2920	End 2956	End 2915	
11	2834–3557	724			Start 2861	Start 2869	Start 2888	
			3010	G	—	—	A	
			3106/3107	C	Del	Del	Del	
					End 3350	End 3373	End 3243	
12	2972–3557	586			Start 2999	Start 2999	Start 3031	
			3106/3107[b]	C	Del	Del	Del	
			3423	G	E	T	T	Silent
					End 3422	End 3460	End 3425	
13	3234–3557	324			Start 3265	Start 3258	Start 3292	
			3423[b]	G	T	T	T	Silent[b]
					End 3548	End 3545	End 3541	
14	3441–3940	500	None		Start 3487	Start 3491	Start 3499	
					End 3916	End 3920	End 3847	
15	3635–4162	528	None		Start 3667	Start 3662	Start 3725	
					End 4126	End 4061	End 4044	
16	3931–4728	798			Start 3964	Start 3968	Start 3987	
			4135	T	—	C	—	Try → His
					End 4399	End 4427	End 4436	
17	4183–4728	546	None		Start 4208	Start 4249	Start 4208	
					End 4657	End 4657	End 4642	
18	4392–4982	591			Start 4449	Start 4453	Start 4440	
			4769	A	G	G	G	Silent
					End 4860	End 4935	End 4877	
19	4447–4982	536			Start 4492	Start 4492	Start 4492	
			4769[b]	A	G	G	G	Silent[b]

TABLE 2—*Continued*

Primer set	Amplified region[a]	Length of amplified region	Comparison with Anderson					Amino acid change
			Anderson No.	Anderson bp	Template CHR	Template 9947A	Template GM03798	
					End 4958	End 4921	End 4931	
20	4797–5553	757			Start 4838	Start 4845	Start 4838	
			4985	G	A	A	A	Silent
			5186	A	G	—	—	Silent
					End 5327	End 5324	End 5215	
21	4976–5553	578			Start 5000	Start 5007	Start 5016	
			5186[b]	A	G	—	—	Silent[b]
					End 5516	End 5521	End 5400	
22	5318–5882	565	None		Start 5361	Start 5360	Start 5371	
					End 5754	End 5758	End 5800	
23	5700–6262	563	None		Start 5741	Start 5744	Start 5754	
					End 6149	End 6163	End 6136	
24	5999–6526	528			Start 6043	Start 6058	Start 6047	
			6221	T	C	—	—	Silent
			6371	C	T	—	—	Silent
					End 6442	End 6503	End 6456	
25	6242–6526	285			Start 6271	Start 6302	Start 6293	
			6371[b]	C	T	—	—	Silent[b]
					End 6520	End 6520	End 6520	
26	6426–7030	605			Start 6451	Start 6474	Start 6487	
			6791	A	G	—	—	Silent
			6849*	A	G (0.3A)[b,*]	—	—	Thr → Ala*
					End 6916	End 6930	End 6885	
27	6744–7255	512			Start 6775	Start 6782	Start 6801	
			6849[b,*]	A	G (0.3A)[b,*]	—	—	Thr → Ala[b,*]
			7028	C	T	—	—	Silent
					End 7215	End 7221	End 7177	
28	7075–7792	718	None		Start 7123	Start 7123	Start 7130	
					End 7602	End 7601	End 7547	
29	7215–7792	578			Start 7263	Start 7280	Start 7273	
			7645	T	—	C	—	Silent
					End 7722	End 7769	End 7706	
30	7645–8215	571			Start 7671	Start 7666	Start 7701	
			7861	T	—	C	—	Silent
					End 8149	End 8155	End 8156	
31	7901–8311	411	None		Start 7960	Start 7959	Start 7960	
					End 8289	End 8288	End 8258	
32	8164–8669	506			Start 8211	Start 8212	Start 8230	
			8448	T	—	C	—	Met → Thr
			8503	T	C	—	—	Silent
					End 8646	End 8641	End 8637	
33	8539–9059	521			Start 8581	Start 8582	Start 8581	
			8860	A	G	G	G	Thr → Ala
					End 9019	End 8999	End 8991	
34	8903–9403	501			Start 8947	Start 8944	Start 8951	
			9315	T	—	C	—	Phe → Leu
					End 9380	End 9381	End 9370	
35	9309–9848	540			Start 9334	Start 9333	Start 9333	
			9559	G	C	C	C	Arg → Pro
					End 9823	End 9827	End 9800	
36	9449–9995	547			Start 9476	Start 9485	Start 9479	
			9559[b]	G	C	C	C	Arg → Pro[b]
					End 9964	End 9940	End 9911	
37	9754–10275	522	None		Start 9777	Start 9781	Start 9808	
					End 10225	End 10251	End 10184	
38	10127–10556	430	None		Start 10168	Start 10166	Start 10180	
					End 10534	End 10536	End 10524	
39	10386–11166	781	None		Start 10410	Start 10416	Start 10439	
					End 10899	End 10916	End 10865	
40	10704–11267	564	None		Start 10734	Start 10742	Start 10758	
					End 11223	End 11197	End 11167	
41	11001–11600	600			Start 11026	Start 11040	Start 11059	
			11335	T	C	C	C	Silent
					End 11461	End 11517	End 11497	

TABLE 2—*Continued*

Primer set	Amplified region[a]	Length of amplified region	Comparison with Anderson					Amino acid change
			Anderson No.	Anderson bp	Template CHR	Template 9947A	Template GM03798	
42	11403–11927	525			Start 11428	Start 11432	Start 11456	
			11719	G	A	—	—	Silent
					End 11795	End 11853	End 11855	
43	11760–12189	430			Start 11784	Start 11802	Start 11802	
			11878	T	C	—	—	Silent
					End 12159	End 12164	End 12163	
44	11901–12876	976	None		Start 11926	Start 11926	Start 11961	
					End 12404	End 12443	End 12397	
45	12357–12876	520			Start 12404	Start 12391	Start 12391	
			12612	A	G	—	—	Silent
			12705	C	T	—	—	Silent
					End 12769	End 12849	End 12775	
46	12601–13123	523			Start 12627	Start 12645	Start 12643	
			12705[b]	C	T	—	—	Silent[b]
					End 13102	End 13045	End 13024	
47	12793–13343	551	None		Start 12817	Start 12807	Start 12816	
					End 13295	End 13307	End 13266	
48	13188–13611	424			Start 13238	Start 13238	Start 13244	
			13572	T	—	C	—	Silent
					End 13587	End 13593	End 13590	
49	13518–13935	418			Start 13541	Start 13541	Start 13571	
			13572[b]	T	—	C	—	Silent[b]
			13702	G	C	C	C	Gly → Arg
			13708	G	A	—	—	Ala → Thr
			13759	G	—	A	—	Ala → Thr
					End 13910	End 13921	End 13900	
50	13715–14118	404			Start 13775	Start 13760	Start 13760	
			13966	A	G	—	—	Thr → Ala
					End 14094	End 14110	End 14104	
51	13899–14388	490			Start 13926	Start 13927	Start 13961	
			13966[b]	A	G	—	—	Thr → Ala[b]
			14199	G	T	T	T	Pro → Thr
			14272	G	C	C	C	Phe → Leu
			14365	G	C	C	E	Silent
					End 14369	End 14374	End 14342	
52	14189–14926	738			Start 14216	Start 14216	Start 14240	
			14272[b]	G	C	C	C	Phe → Leu[b]
			14365[b]	G	C	C	C	Silent[b]
			14368	G	C	C	C	Phe → Leu
			14470	T	C	—	—	Silent
			14766	T	E	C	E	Ile → Thr
					End 14699	End 14806	End 14698	
53	14470–14996	527			Start 14502	Start 14513	Start 14527	
			14766[b]	T	—	C	C	Ile → Thr[b]
					End 14957	End 14972	End 14956	
54	14909–15396	488			Start 14941	Start 14933	Start 14950	
			15326	A	G	G	G	Thr → Ala
					End 15380	End 15373	End 15359	
55	15260–15774	515			Start 15305	Start 15293	Start 15287	
			15326[b]	A	G	G	G	Thr → Ala[b]
			15646	C	—	—	T	Silent
					End 15754	End 15950	End 15723	
56	15574–16084	511			Start 15637	Start 15599	Start 15601	
			15646[b]	C	—	—	T	Silent[b]
					End 16056	End 16058	End 16030	
57 (HV1)	15971–16451	481			Start 16014	Start 16011	Start 16004	
			16183	A	C	—	—	
			16189	T	C	—	—	
			16311	T	E	C	—	
			16357	T	E	—	C	
					End 16193	End 16430	End 16403	
58	16097–336	809			Start 16125	Start 16130	Start 16151	
			16183[b]	A	C	—	—	
			16189[b]	T	C	—	—	

TABLE 2—*Continued*

Primer set	Amplified region[a]	Length of amplified region	Comparison with Anderson: Anderson No	Anderson bp	Template CHR	Template 9947A	Template GM03798	Amino acid change
			16311[b]	T	E	C	—	
			16357[b]	T	E	—	C	
			16519	T	E	C	C	
					End 16193	End 59	End 103	
21M13[c] cloned DNA	16133–40	477			Start 16131			
			16183[b]	A	C	ND	ND	
			16189[b]	T	C			
			16193 1		C(ins)			
			16223	C	T			
			16278	C	T			
			16519[b]	T	C			
					End 40			

Note. B, basepair change came before the readable sequence; E, basepair change came after the readable sequence; —, basepair same as in Anderson sequence, h*, possible heteroplasmic site. * This heteroplasmy seen in the first CHR cell culture line was not seen with the second CHR cell culture line. It is the second CHR cell culture line that is supplied in NIST SRM 2392, Start, start of readable sequence, End, end of readable sequence; CHR cells, sequence based on two amplifications and cycle sequencing procedures in first cell culture line and at least one amplification and cycle sequencing procedure with the second cell culture line, 9947A cells, sequence based on two amplifications and cycle sequencing procedures; GM03798, sequence based on three to four amplifications and cycle sequencing procedures; ins, insertion; Del, deletion; ND, not done

[a] Numbers correspond to Anderson sequence (Anderson *et al.*, 1981).

[b] Change also seen in previous primer set

[c] This primer is used for sequencing the cloned DNA of the HV1 region

the entire mtDNA were completely amplified and sequenced at least twice (GM03798 was done three times). There were 13, 9, and 4 differences in the noncoding regions of templates CHR, 9947A, and GM03798, respectively, and 33, 23, and 19 differences in the coding regions of templates CHR, 9947A, and GM03798, respectively. All of the differences from Anderson found in these three templates are shown in Fig. 2 along with many of the diseases that have been noted in the literature (Wallace *et al.*, 1997). None of the basepair changes found in the coding regions of the three templates sequenced at NIST correlate with any of the changes found associated with these published disease states.

Meaning of the differences from Anderson. Since all three templates had come from apparently normal individuals, it was of interest to determine if the differences in the coding regions would actually cause amino acid changes in the resultant protein structures. The genetic code for human mtDNA is slightly different from the universal genetic code (Anderson *et al.*, 1981). One needs to consider these differences in the universal genetic code when determining the amino acid sequence designated by the 3-bp codons in mtDNA. Many of the differences from the Anderson sequence were in the third position wobble and did not affect the amino acid sequence (silent changes) (Table 2). However, CHR, 9947A, and GM03798 had 10 (9 without the heteroplasmy at bp 6849), 12, and 8 different basepairs, respectively, that would result in a different amino acid from that designated by the Anderson sequence (Table 2). However, data from the literature indicate that perhaps 14 basepair designations in the consensus sequence of Anderson may not be the sequence found in the majority of normal individuals (Howell *et al.*, 1992; Marzuki *et al.*, 1992). Our results agree with 11 of these 14 new designations (Table 3). Examination of our results using these new designations indicates that only 4 (3 without the heteroplasmy at bp 6849), 6, and 2 differences for CHR, 9947A, and GM03798, respectively, would result in amino acid changes. These structural changes, however, do not necessarily mean a functional change has occurred in the protein. To determine if a functional change has occurred, one still needs to decipher whether the amino acid change is in an active site on the protein.

The interlaboratory evaluation of the CHR template. An interlaboratory evaluation was conducted by four laboratories including NIST. All of the laboratories essentially followed the NIST protocol sent with the DNA from the first CHR cell culture line and the primers. Any changes to the protocol are listed under Materials and Methods. Each laboratory was instructed to amplify and sequence the 58 areas designated by the 58 primer sets and also to sequence the cloned DNA for the HV1 region. Laboratory 1 amplified and sequenced each area at least twice. The other labs amplified and sequenced the areas from one to six times. Laboratories 1, 2, and 3 found essentially the same polymorphisms. Laboratory 4 had less experience with sequencing mtDNA and did find differences that the other laboratories did not observe. Data were excluded

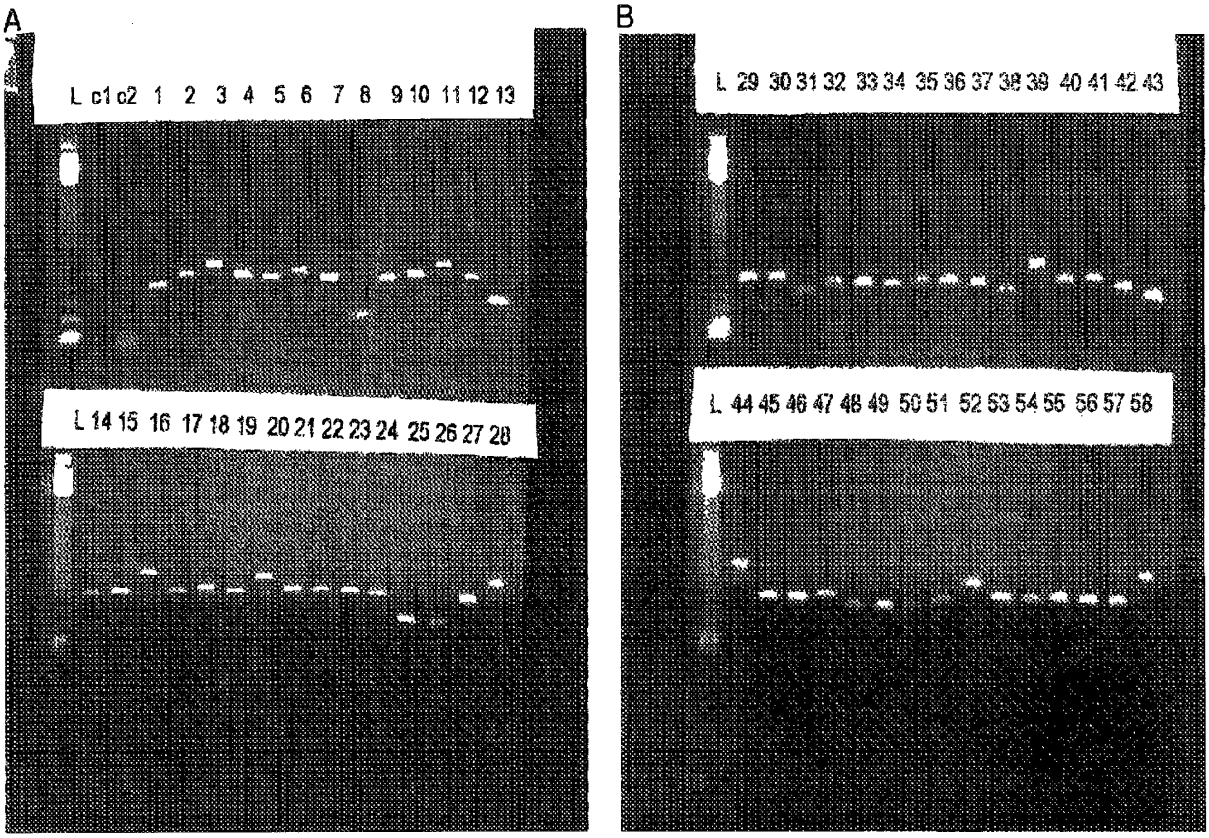

FIG. 1. Agarose gel electrophoresis of PCR products from 58 primer sets designed for human mitochondrial DNA (**A**) Lane **L,** 123-bp ladder from Gibco BRL, lane **c1,** negative control with no primers, lane **c2,** negative control with no DNA, lanes **1–28,** PCR products from primer sets 1–28 (**B**) Lane **L,** 123-bp ladder from Gibco BRL; lanes **29–58,** PCR products from primer sets 29–58.

from the analysis of this interlaboratoy evaluation if the following conditions were observed: (1) The computer results were ambiguous as indicated by calling a peak "N" rather than A, C, G, or T. (2) The differences from Anderson were not consistently seen within a laboratory, i.e., if the laboratory sequenced in both the forward and the reverse directions and one direction agreed with Anderson and the other direction did not agree, we assumed the results that agreed with Anderson were correct. (3) Within any one laboratory, the difference from Anderson was seen with one primer set, but not in the overlapping sequences seen in the previous or subsequent primer sets. Even with these exclusions, Laboratory 4 had many differences that were not seen by the other labs. One problem was that they did not provide data from primer sets 29, 39, and 41, and we were unable to check those overlapping sequences. Laboratory 2 was unable to sequence the clone, which was not a problem for the other laboratories. Laboratory 3 was missing data from primer sets 36 and 48. Laboratories 1 and 3 noted a heteroplasmy[5] at base number 6849 (Anderson found an A at this site). Laboratory 1 found a G at this site, but closer examination of the electropherogram showed that an A peak existed under the G peak. Laboratory 3 also noted the A/G heteroplasmy at this site. Laboratory 4 did not note the heteroplasmy, but when their electropherograms were examined at NIST, the A/G heteroplasmy was noted. NIST did not have the electropherograms of Laboratory 2, but on questioning them, they agreed that the heteroplasmy was there, but that they had missed it. One of the problems with finding heteroplasmic sites is that if the computer call is the same as Anderson, one would not necessarily examine that site more closely. If the computer call is different from Anderson, one would look more closely at the electropherogram and then note the presence of a smaller peak under the main peak.

With the exceptions of the differences noted here, the interlaboratory evaluation was successful in that most of the laboratories found the same results. The many differences noted by Laboratory 4, which was less experienced at sequencing mtDNA, confirm and emphasize the need for a standard reference mate-

[5] In final preparation of this SRM, a new blood sample was obtained from CHR and a new cell culture line was established. The sequence analysis of the new CHR was identical to the first cell line except no heteroplasmy was found at bp 6849. Therefore, the cell line supplied with this SRM does not have this heteroplasmy. The data on the first cell line are included in the text to indicate the agreement in the interlaboratory evaluation that was done with the first cell line

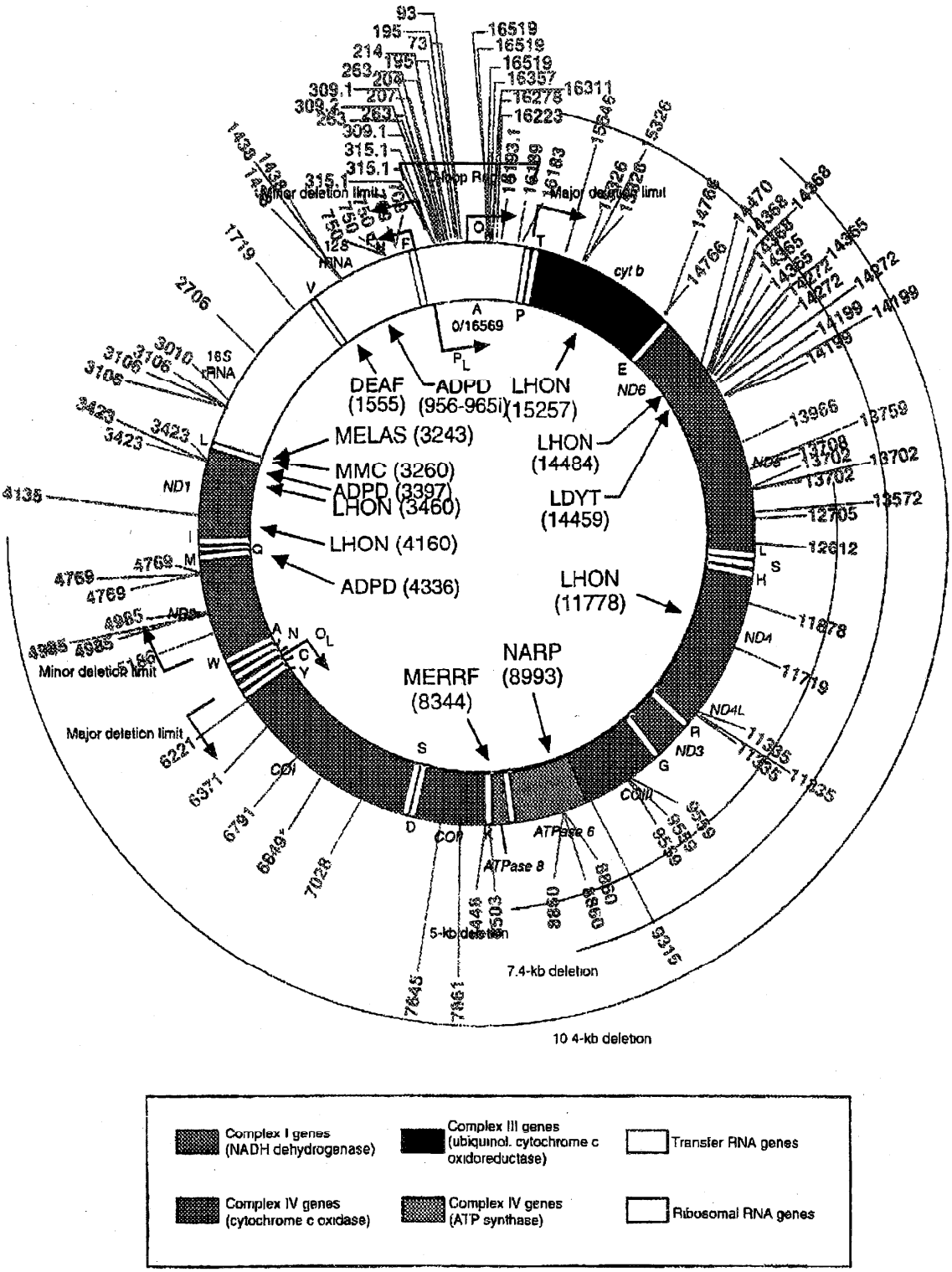

FIG. 2. Human mitochondrial DNA (color-coded circle) showing positions of genes, diseases, and deletions (Wallace, 1992; Wallace *et al.*, 1997) and the polymorphisms found in three apparently normal individuals examined for this study. The pink (CHR), green (9947A), and blue (GM03798) numbers and lines along the outside of the color-coded circle indicate the locations of differences from Anderson found in this study. Locations of disease base substitutions are shown on the inside of the circle. Abbreviations: ADPD, Alzheimer disease and/or Parkinson disease; DEAF, neurosensory hearing loss, LDYT, LHON plus dystonia; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fiber disease; MMC, myopathy and cardiomyopathy; NARP, neurogenic muscle weakness, ataxia, and retinitus pigmentosum. Large deletions are shown by the concentric black semicircles along the outside of the colored circle. tRNA genes (white areas in color-coded circle) are indicated by their amino acid single-letter abbreviation. Modified with permission of the publisher from Wallace (1992) where additional information on this figure may be found 6849*, see legend to Table 2.

TABLE 3

Errors vs Polymorphisms in mtDNA Sequence Determined by Anderson

Basepair	Anderson designation → literature designation (No. found/No. examined)	Anderson designation → NIST mt SRM designation (No. found/No. examined)	Change
1438	A → G[a]	A→G (3/3)	12 s rRNA
3423	G → T (87/87)[b]	G→T (3/3)	SILENT
4769	A → G (28/30)[b]	A→G (3/3)	SILENT
4985	G → A (9/9)[b]	G→A (3/3)	SILENT
8860	A → G[a]	A→G (3/3)	Thr → Ala
11335	T → C (8/8)[b]	T→C (3/3)	SILENT
11719	G → A (26/37)[b]	G→A (1/3)	SILENT
12308	A → G (3/9)[b]	Change not found	$tRNA^{leu}$
13702	G → C (105/105)[b]	G→C (3/3)	Gly → Arg
14199	G → T (9/9)[b]	G→T (3/3)	Pro → Thr
14272	G → C (9/9)[b]	G→C (3/3)	Phe → Leu
14365	G → C (9/9)[b]	G→C (3/3)	SILENT
14368	G → C (9/9)[b]	G→C (3/3)	Phe → Leu
15326	A → G (6/6)[b]	A→G (3/3)	Thr → Ala

Note. Anderson *et al.* (1981) sequenced mtDNA mainly from a single human placenta, although some regions were from HeLa cells. Five ambiguous regions (bp 10, 934, 935, 14272, 14365) were assumed by Anderson to be same as that found in bovine mtDNA.

[a] Marzuki *et al.*, 1992

[b] Howell *et al.*, 1992.

rial for sequencing mtDNA. If Laboratory 4 had the NIST mtDNA SRM 2392 and had run it alongside their unknown sample, they would have realized that they were finding an undue number of differences and could have reexamined their procedures to try to determine the reason for these excessive changes.

CONCLUSIONS

A NIST standard reference material (SRM 2392) that allows one to sequence any region or the entire 16,569 bp that comprise human mtDNA has been prepared. Fifty-eight pairs of unique primers have been designed, tested, and shown to work well in the amplification and sequencing procedures. The two DNA templates (CHR and 9947A) included in the SRM have characteristic polymorphisms throughout the noncoding and coding regions of the DNA and, therefore, can serve as positive controls during PCR amplification and sequencing. None of these polymorphisms correspond to any of the published basepair changes that have been correlated with specific diseases.

Compared to the Anderson sequence, CHR mtDNA had 13 differences in the noncoding regions and 33 differences in the coding regions, and the 9947A mtDNA had 9 differences in the noncoding regions and 23 differences in the coding regions. GM03798, whose data are included for comparison and information, had 4 differences in the noncoding regions and 19 differences in the coding regions. These differences in the coding regions do result in some amino acid changes in the proteins coded for by mtDNA. Four laboratories participated in an interlaboratory evaluation of the CHR template; some differences between laboratories were noted, but, in general, agreement was good. The use of NIST SRM 2392 will provide quality control to the scientific and medical communities when they sequence human mtDNA.

ACKNOWLEDGMENTS

The authors are grateful to The Bode Technology Group, Inc. (Sterling, VA), IIT Research Institute (Newington, VA), and Lark Technologies, Inc. (Houston, TX) for their participation in the interlaboratory evaluation. The authors also acknowledge the funding received from the National Institute of Justice through the NIST Office of Law Enforcement Standards and the support from the Advanced Technology Program at NIST. The authors thank Prasad Reddy for his help and advice on cloning, MaryPat Jones for confirming the sequences of some areas of the second CHR cells, and Catherine O'Connell for the use of her computer with the Gene Runner Program and her advice in the use of the program.

REFERENCES

Anderson, S., Bankier, A. T., Barrell, B. G., deBrujin, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature* **290:** 457–465.

Ballinger, S. W., Bouder, T. G., Davis, G. S., Judice, S. A., Nicklas, J. A., and Albertini, R. J. (1996). Mitochondrial genome damage associated with cigarette smoking. *Cancer Res.* **56:** 5692–5697

Bendall, K. E., and Sykes, B. C. (1995). Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. *Am J Hum Genet.* **57:** 248–256.

Bogenhagen, D., and Clayton, D. A. (1974). The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells *J Biol Chem* **249:** 7991–7995.

Grossman, L. I (1995) Mitochondrial mutations and human disease. *Environ Mol. Mutagen.* **25**(Suppl. 26): 30–37.

Holland, M. M., Fisher, D. L., Mitchell, L. G., Rodriquez, W. C., Canik, J. J., Merril, C. R., and Weedn, V. W. (1993). Mitochondrial DNA sequence analysis of human skeletal remains: Identification of remains from the Vietnam War. *J. Forensic Sci.* **38:** 542–553.

Holland, M M., Fisher, D. L., Roby, R. K., Ruderman, J , Bryson, C., and Weedn, V. W (1995). Mitochondrial DNA sequence analysis of human remains. *Crime Lab. Dig.* **22:** 109–115.

Howell, N , McCullough, D. A., Kubacka, I., Halvorson, S., and Mackey, D (1992). The sequence of human mtDNA: The question of errors versus polymorphisms. *Am. J. Hum. Genet.* **50:** 1333–1337.

King, M P., and Attardi, G. (1989). Human cells lacking mtDNA: Repopulation with exogenous mitochondria by complementation. *Science* **246:** 500–503

Levin, B C., Cheng, H., O'Connell, C., Reeder, D. J., and Holland, M. (1995). Evaluation of alternative enzymes and additives to improve sequencing of mitochondrial DNA. Proc. Fifth Int. Symp. on Human Identification, Scottsdale, Arizona, October 8–12, 1994, p. 170

Levin, B. C., Cheng, H., Holland, M., and Reeder, D J (1997). Heteroplasmy responsible for difficulty experienced in sequencing the human mitochondrial DNA HV1 region containing the C-stretch Proc. Seventh Int. Symp. on Human Identification, Scottsdale, Arizona, September 19–21, 1996, p. 166.

Marzuki, S., Lertrit, P., Noer, A. S , Kapsa, R M I., Sudoyo, H., Byrne, E , and Thyagarajan, D. (1992) Reply to Howell *et al.*: The need for a joint effort in the construction of a reference data base for normal sequence variants of human mtDNA *Am. J Hum. Genet.* **50:** 1337–1340

Sambrook, J , Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wallace, D. C. (1992). Mitochondrial genetics: A paradigm for aging and degenerative diseases. *Science* **256:** 628–632

Wallace, D. C., Brown, M. D., and Lott, M. T. (1997) Mitochondrial genetics *In* "Emery and Rimoin's Principles and Practice of Medical Genetics" (D. L. Rimoin, J. M Connor, and R. E. Pyeritz, Eds.), 3rd ed., Vol. 1, pp. 277–332, Churchill Livingstone, New York